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(54) **CRYOPRESERVATION OF DIVERSE PLANT CELLS**

TIEFKÜHLKONSERVIERUNG VON VERSCHIEDENEN PFLANZENZELLEN

CRYOCONSERVATION DE CELLULES VEGETALES DIVERSES

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established callus. Observations on viability of cells were made at different times after thawing, but the most important observation was generally recorded. Cells on a filter paper were transferred to a normal solid medium without the presence of any additional osmotic agent. Cell growth recovery rate was observed during the first 3 weeks of incubation of all biomass on a filter in the dark at 25°C.

[0134] An alternate procedure related to the post-thawing of cells or cell biomass was also useful. This procedure initially involved washing the cells quickly with a liquid medium containing an osmotic agent (sucrose, sorbitol or mannitol) at a concentration ranging from 1-2M without ethylene inhibitor. This was done by incubation of cell biomass for 2-5 minutes in liquid nutrient medium containing an osmotic agent and centrifugation at 100 g for 1-3 minutes. This step was repeated twice to remove toxic cryoprotectants from the vitrifying solution followed by incubation of cell biomass for 30 minutes in liquid nutrient-medium containing an osmotic agent and an ethylene inhibitor (concentration range: 2-30 uM).

[0135] Ethylene inhibitors are substances that interfere with ethylene production, metabolism or ethylene action. They may be further classified as ethylene-biosynthesis antagonists and ethylene-action antagonists. Ethylene-biosynthesis agonists are compounds that interfere with the biosynthetic pathway to ethylene. Examples of enzymes along this biosynthetic pathway that are inhibited include ACC synthase, ACC oxidase and ethylene oxidase. Examples of ethylene biosynthesis antagonists include α-aminoisobutyric acid, acetylsalicylicacid, methyoxyvinylglycine, aminooxyacetic acid and the like. Examples of ethylene action antagonists include silver containing compounds, silver complexes or silver ions, carbon dioxide, 1-methylcyclopropene, 2,5-norbornadiene, trans-cyclooctene, cisbutene, diazocyclopentadiene and the like. Suitable silver salts include silver nitrate, silver thiosulfate, silver phosphate, silver benzoate, silver sulfate, silver salt of toluenesulfonic acid, silver chloride, silver oxide, silver acetate, silver pentafluoropropionate, silver cyanate, silver salt of lactic acid, silver hexafluoropropionate, silver cyanate, silver salt of lactic acid, silver hexafluorophosphate, silver nitrite, and the trisilver salt of citric acid. Illustrative examples of the enhancement of taxane biosynthesis by a variety of silver salts are shown in Tables 1 and 2.

[0136] Experimental conditions consisted of nutrient liquid medium containing 1.25M of an osmotic agent and the appropriate concentration of SLTS. The osmotic agent was sucrose, sorbitol or mannitol. Control conditions consisted of nutrient liquid medium containing 1.25M of an osmotic agent but without SLTS. Results are shown in Table 21 and indicate the extent of callus cell growth and percent recover rate.

Table 21

Influence of Post-thaw Treatment with Ethylene Inhibitor - Silver Thiosulfate (SLTS) on Viability and Recovery Regrowth of Cells

Plant Species	Control	Percent Viability and Cell Growth Intensity Experimental-SLTS Concentration (μM)				
	(No SLTS)	2 Day	4 Day	8 Day	10 Day	20 Day
Taxus sp	35(+)	40(+)	50(++)	80(+++)	60(+++)	30(+)
Lycopersicum sp	40(+)	45(++)	60(++)	90(+++)	80(+++)	40(+)

(+) = Moderate recovery regrowth with visible cell growth in two weeks
(++) = Vigorous recovery regrowth with visible cell growth in 6-10 days
(+++)= Vigorous recovery regrowth with visible cell growth in 3-4 days

[0137] Post-thaw treatment of cells with liquid medium containing ethylene inhibitor consistently improved the viability and growth rate of recovered cell lines. Silver thiosulfate at concentrations ranging from 4μM - 10 μM was more effective than at 2 μM and 20 μM for enhancing growth rates of recovered cell lines. Viability rates up to 90% were achieved depending upon the cell line used. Post-thaw liquid medium containing SLTS at 8 μM and 10 μM promoted cell growth much more vigorously, and cell growth was macroscopically visible 3-4 days after plating cells on nutrient medium free from osmotic agent and ethylene inhibitor.

Claims

1. A method for cryopreserving a plant cell comprising a combination of treatments as follows:

   a. treating the plant cell by lyophilizing the plant cell, by heat shock or by pretreating the plant cell with a cryoprotective agent and a stabilizer which is an anti-oxidant, an oxygen-radical scavenger, a divalent cation,

an ethylene inhibitor, a compound that intercalates into the lipid bilayer of the cell (for example a sterol, a phospholipid, a glycolipid or a glycoprotein) or a combination thereof;
b. vitrifying the plant cell in a vitrifying solution; and
c. freezing the vitrified plant cell at a cryopreservation temperature.

2. The method of claim 1, in which method:

(i) the treating comprises pretreatment with a cryoprotective agent and a stabiliser and the cryoprotective agent comprises a substance selected from the group consisting of DMSO, propylene glycol, glycerol, polyethylene glycol, ethylene glycol, butanediol, formamide, propanediol, sorbitol, mannitol and mixtures thereof; or
(ii) the vitrifying solution comprises a substance selected from said group.

3. The method of claim 1 or claim 2 wherein the treating comprises pretreatment with a cryoprotective agent and a stabilizer selected from reduced glutathione, tetramethylurea, tetramethylthiourea, dimethylformamide, mercaptopropionyl glycine, mercaptoethylamine, selenomethionine, thiourea, dimercaptopropanol, ascorbic acid, cysteine, sodium diethyldithiocarbamate, sodium thiosulfate, silver thiosulfate, propylgallate, spermine, spermidine and combinations and derivatives thereof.

4. The method of claim 1 or claim 2 wherein the treating comprises pretreatment with a cryoprotective agent and a stabiliser or the method of claim 3, in which method the stabilizer is employed as an aqueous solution at a concentration of from about 1 μM to about 10 mM.

5. The method of claim 1 or claim 2 wherein the treating comprises pretreatment with a cryoprotective agent and a stabiliser or the method of claim 3 or claim 4, in which method the treating is performed at a reduced temperature for a first period of time and the cryoprotective agent comprises a vitrifying agent, and wherein the step of vitrifying comprises incubating the plant cell in the vitrifying solution containing an increased concentration of the vitrifying agent for a second period of time.

6. The method of claim 5 wherein the first period of time is from about one hour to about 7 days and/or the second period of time is from about 30 minutes to about 2 hours.

7. The method of claim 1 wherein the lyophilisation or heat shock is preceded by culturing the cell with an osmotic agent, the osmotic agent optionally comprising fructose, glucose, maltose, mannitol, sorbitol, sucrose, trehalose and/or proline.

8. The method of any of claims 1 to 7 which further comprises loading the plant cell with a loading agent prior to freezing it.

9. The method of claim 8 wherein loading and vitrifying are performed substantially simultaneously.

10. The method of any of claims 1, 2 or 4 to 9 wherein the pretreatment with a cryoprotective agent and a divalent cation involves addition of the divalent cation into the vitrifying solution.

11. The method of any of claims 1 to 10 which further comprises the step of acclimating the stabilised plant cell to a reduced temperature, the reduced temperature optionally being from about I °C to about 15°C.

12. The method of claim 11 wherein pretreatment involves culturing said plant cell in medium containing a loading agent and said stabilizer for between about 1 hour to about 7 days at about room temperature.

13. The method of any of claims 8, 9 or 12 or of claims 10 or 11 when dependent on claims 8 or 9 wherein the loading agent is a sugar, an amino acid or a combination thereof, the sugar optionally comprising one or more sugars selected from fructose, glucose, maltose, mannitol, sorbitol, sucrose, trehalose and derivatives thereof.

14. The method of any of claims 1 to 13 wherein loading and/or vitrifying is/are performed (i) in a single step or (ii) in a plurality of steps, the plurality of steps optionally comprising adding a cryoprotecting agent to the plant cell five times at one minute intervals.

15. The method of any of claims 1 to 14 wherein the plant cell is a gymnosperm cell, or is an angiosperm cell which

is a monocotyledon plant cell or a dicotyledon plant cell, the monocotyledon plant cell optionally being selected from the group consisting of species of the genus *Avena, Cocos, Dioscorea, Hordeum, Musa, Oryza, Saccharum, Sorghum, Triticum* and *Zea.*

16. The method of claim 15 wherein the dicotyledon plant cell is selected from the group consisting of species of the genus *Achyrocline, Atropa, Brassica, Berberis, Capsicum, Catharanthus, Conospermum, Datura, Daucus, Digitalis, Echinacea, Eschscholtzia, Glycine, Gossypium, Hyoscyamus, Legume, Lupinus, Lycopersicum, Malus, Medicago, Nicotiana, Panax, Pisum, Rauvolfia, Ruta, Solanum, Sophora* and *Trichosanthes,* and the gymnospenn is a species of *Abies, Cypressus, Ginkgo, Juniperus, Picea, Pinus, Pseudotsuga, Sequoia, Taxus, Tsuga* or *Zamia.*

17. The method of claim 16 wherein the *Taxus* species is T. *baccata, T. brevifolia, T. canadensis, T. chinensis, T cuspidata, T floridana, T globosa, T media, T nucifera* or *T wallichiana.*

18. The method of any of claims 1 to 17 wherein the plant cell is obtained from new growth needles, bark, leaves, stem, root, rhizome, callus cells, protoplasts, cell suspensions, meristems, seeds or embryos.

19. The method of any of claims 1 to 18, wherein greater than about 50% of plant cells cryopreserved by the method are capable of being recovered in a viable state and optionally greater than 70% or greater than 80% of such plant cells are capable of being recovered in a viable state.

20. The method of claim 19 wherein said cells are not significantly genetically or phenotypically altered by the cryopreservation.

21. The method of claim 19 or claim 20 wherein the plant cells are of a Taxus species, the *Taxus* cell optionally expressing a diterpenoid, particularly taxol.

22. The method of claim 21 wherein expression of said diterpenoid is not significantly altered by cryopreservation.

23. The method of any of claims 1 to 18 which comprises forming a germ cell bank of the cryopreserved plant cells , greater than about 50% of the plant cells being capable of being recovered in a viable state and optionally greater than 70% or greater than 80% of the plant cells being capable of being recovered in a viable state, .

24. A method for recovering cryopreserved plant cells comprising a combination of treatments as follows:

a) thawing the cryopreserved plant cells to a temperature above freezing;
b) incubating the thawed plant cells in a growth medium containing a stabilizer or a cryoprotective agent and a stabiliser, which stabiliser is an anti-oxidant, an oxygen-radical scavenger, an ethylene inhibitor, a compound that intercalates into the lipid bilayer of the cell (for example a sterol, a phospholipid, a glycolipid or a glycoprotein), a divalent cation or a mixture thereof; and
c) recovering viable plant cells.

25. The method of claim 24 wherein the cryoprotective agent is

(i) a sugar, an amino acid or a mixture thereof; or

(ii) is selected from sorbitol, mannitol, sucrose, trehalose, proline and mixtures thereof.

26. The method of claim 24 or claim 25 which further includes, before step (c), the step of removing the cryoprotective agent, and optionally wherein the removal step comprises multiple washings of osmotically adjusted cells with said growth medium containing decreasing concentrations of said cryoprotective agent and/or wherein the cryoprotective agent is removed after a period of time and incubation of plant cells is continued in growth medium and in suspension.

27. The method of any of claims 24 to 26 wherein the ethylene inhibitor is

(i) an ethylene biosynthesis inhibitor, which is optionally selected from spermidine, spermine, catechol, n-propyl gallate, hydroquinone, ferulic acid, alar, phenylethylamine, salicyl alcohol, indomethacin and combinations thereof; or

(ii) an ethylene action inhibitor, which ethylene action inhibitor is optionally a silver salt and the silver salt is optionally selected from silver thiosulfate, silver nitrate, silver chloride, silver acetate, silver phosphate, silver sulfate, silver nitrite and combinations thereof.

28. The method of any of claims 24 to 27 wherein the divalent cation is calcium, magnesium or manganese.

29. The method of any of claims 24 to 28 wherein the stabilizer is selected from reduced glutathione, tetramethylurea, tetramethylthiourea, dimethylformamide, mercaptopropionyl glycine, mercaptoethylamine, selenomethionine, thiourea, dimercaptopropanol, sodium thiosulfate, silver thiosulfate, ascorbic acid, cysteine, sodium diethyldithiocarbamate, spermine, spermidine, propylgallate and combinations and derivatives thereof.

30. The method of any of claims 24 to 29 wherein the incubating and recovering are performed in a liquid medium.

31. The method of any of claims 24 to 29 wherein the incubating is performed on a semi-solid medium.

32. The method of any of claims 24 to 29 wherein the thawed plant cells are incubated in suspension and the viable plant cells are recovered in suspension.

33. The method of any of claims 24 to 32 wherein the recovered plant cells are transferred to a semi-solid medium.

34. The method of any of claims 24 to 33 wherein the recovered viable plant cells express a diterpenoid and diterpenoid expression is not significantly altered by cryopreservation and/or wherein greater than about 50% of recovered plant cells are viable, and optionally wherein greater than about 70% of recovered plant cells are viable or greater than about 80% of recovered plant cells are viable.

35. The method of any of claims 24 to 34 further comprising the step of cryopreserving the plant cells according to the method of any of claims 1 to 18.

36. The method of any of claims 24 to 35 wherein of the plant cells recovered greater than about 50% are viable, and optionally of which greater than about 70% are viable or of which greater than about 80% are viable.

37. The method of claim 36 wherein the plant cells are a species of *Taxus* and/or are in suspension.

38. A method of producing a plant product, comprising:

A) performing the method of any of claims 1 to 18 and recovering a viable plant cell, or performing the method of any of claims 24 to 35;
B) propagating cells or plants from the resultant viable cell; and
C) collecting a plant product produced by the propagated material.

39. The use of the method of claim 38 to produce a diterpenoid, the diterpenoid optionally being taxol for chemotherapeutic use.

Patentansprüche

1. Verfahren zur Tiefkühlkonservierung einer Pflanzenzelle, umfassend eine Kombination von Behandlungsschritten wie folgt:

(a) Behandlung der Pflanzenzelle durch Lyophilisieren der Pflanzenzelle, mittels Wärmeschock oder durch Vorbehandeln der Pflanzenzelle mit einem Mittel zum Schutz beim Tiefgefrieren und einem Stabilisator, der ein Oxidationsinhibitor, ein Fänger für Sauerstoffradikale, ein zweiwertiges Kation, ein Ethyleninhibitor, eine Verbindung, die sich in die Lipid-Doppelschicht der Zelle interkaliert (beispielsweise ein Sterol, ein Phospholipid, ein Glycolipid oder ein Glycoprotein) oder eine Kombination daraus ist;
(b) Vitrifizieren der Pflanzenzelle in einer vitrifizierenden Lösung; und
(c) Frieren der vitrifizierten Pflanzenzelle bei einer Tiefkühlkonservierungs-Temperatur.

2. Verfahren nach Anspruch 1, worin